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## Correspondence

# First survey of the pathogenic fungus *Batrachochytrium salamandrivorans* in wild and captive amphibians in the Czech Republic

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The recently discovered fungal pathogen *Batrachochytrium salamandrivorans* (Martel et al. 2013) (hereinafter referred to as *Bsal*) has already received significant scientific and public attention (e.g., Martel et al. 2014, Van Rooij et al. 2015, Yap et al. 2015, Stegen et al. 2017). The *Bsal* epidemic has so far been limited to European newts and salamanders found in the wild (Belgium, Germany and the Netherlands: Martel et al. 2013, Spitzen-van der Sluijs et al. 2016) and in captive populations (Germany: Sabino-Pinto et al. 2015; United Kingdom: Cunningham et al. 2015). In the Netherlands, *Bsal* is responsible for the near extinction of wild fire salamander (*Salamandra salamandra*) populations (Spitzen-van der Sluijs et al. 2013).

The Bern Convention Standing Committee has therefore announced Recommendation No. 176 on the prevention and control of the *Bsal* chytrid fungus. According to this recommendation, European countries should adopt measures that include establishment of monitoring programmes to control the possible further spread of the disease, especially in areas of high risk (e.g., areas near disease outbreaks), and develop emergency action plans that will allow prompt responses in case of *Bsal* occurrence (Council of Europe 2015).

The Czech Republic is a country with relatively high caudate species diversity (Sillero et al. 2014) and shares a western border with Germany, a country with previously proven *Bsal* occurrence (Sabino-Pinto et al. 2015, Spitzen-van der Sluijs et al. 2016). The country, and especially the capital city of Prague, has an active and sizeable community of exotic pet keepers and pet shops, and large exotic pet fairs take place on a regular basis (Havlíková et al. 2015). Furthermore, Prague and its surroundings are known to harbour wild populations of at least four native caudate species: fire salamander, smooth newt (*Lissotriton vulgaris*), alpine newt (*Ichthyosaura alpestris*) and great crested newt (*Triturus cristatus*) (Šťastný et al. 2015). All four are susceptible to *Bsal*-induced mortality (Martel et al. 2013, 2014, Cunningham et al. 2015, Spitzen-van der Sluijs et al. 2016, Stegen et al. 2017). The surrounding areas of such large cities are likely to constitute areas of high risk for wild populations of native amphibians. For this reason, we selected Prague and its surroundings as the first focal area for *Bsal* surveillance efforts in wild populations of Czech caudate amphibians. Considering that *Bsal* is spread through the pet trade in caudates originating in Asia (Martel et al. 2014), we also focused on captive collections of caudate amphibians.

In total, 324 swab samples were tested for both *Batrachochytrium dendrobatidis* (*Bd*) and *Bsal* presence and prevalence. In wild populations, 126 samples of three caudate species (fire salamanders, smooth newts and alpine newts) were collected at nine sites within Prague's urban area and its surroundings during autumn 2015 and spring 2016 (Table 1). Furtheremore, we analysed samples from five captive collections, including those of four private breeders and Prague's zoological garden during the period 2015–2016. Within each collection, only subset of about two to four individuals were sampled from an aquarium. This *Bsal*-targeted sampling in captivity was extended by re-analysing samples of caudates from previous surveillance projects

Table 1. Numbers (No.) of individuals sampled within nine wild caudate populations in Prague and its surroundings. Date = date of sampling.

| Locality name | Coordinates | | Species | No. | Date |
|---|---|---|---|---|---|
| Podhořský potok, small stream, tributary of Vltava River | 50.129947°N | 14.404111°E | *Salamandra salamandra* | 31 | 07.10.2015 |
| Únětice, unnamed tributary of Únětický potok | 50.144853°N | 14.384502°E | *Salamandra salamandra* | 25 | 15.10.2015 |
| Levý Hradec, unnamed tributary of Vltava River | 50.169883°N | 14.377429°E | *Salamandra salamandra* | 12 | 20.10.2015 |
| Úholičky, unnamed tributary of Podmoráňský potok | 50.170698°N | 14.344784°E | *Salamandra salamandra* | 8 | 09.11.2015 |
| Lhotecký potok, tributary of Vltava River | 49.956059°N | 14.411423°E | *Salamandra salamandra* | 7 | 15.10.2015 |
| Chalupecká strouha, near confluence with Zvolský potok | 49.930541°N | 14.390361°E | *Salamandra salamandra* | 1 | 17.11.2015 |
| Baně, unnamed tributary of Vltava River | 49.961229°N | 14.392828°E | *Salamandra salamandra* | 2 | 17.11.2015 |
| Ohrobecké údolí, unnamed tributary of Vltava River | 49.943775°N | 14.413338°E | *Salamandra salamandra* | 10 | 21.10.2015 |
| Botanická zahrada, artificial pond in botanic garden | 50.070429°N | 14.421077°E | *Lissotriton vulgaris* | 28 | 01.07.2016 |
| Botanická zahrada, artificial pond in botanic garden | | | *Ichthyosaura alpestris* | 2 | 01.07.2016 |

searching for *Bd* presence in captive amphibians (Havlíková et al. 2015), including 18 individuals of the largest amphibian species, the Chinese giant salamander (*Andrias davidianus*), reared in Prague's zoological garden. In total, 198 samples of 60 caudate (sub)species were analysed in captive collections (Table 2).

Sampling and DNA extraction were performed according to procedures used in amphibian chytridiomycosis research (Boyle et al. 2004). The first sample subset, consisting of 98 wild and 56 captive samples, was checked for *Bsal* presence by SYBRGreen quantitative polymerase chain reaction (qPCR) following the method described in Blooi et al. (2013) as one possible detection option. Bovine serum albumin (BSA) was added to reduce PCR inhibition (Garland et al. 2010).The identity of amplified DNA was checked by melt curve analysis and compared to results for genomic standards of *Bsal* provided by An Martel (Ghent University). We later adopted the duplex *Bd*+*Bsal* qPCR (Blooi et al. 2013) and used it for additional samples. In this assay, we used genomic standards of *Bd* equivalent to 0.1, 1, 10 and 100 zoospores per 5 µl (strain IA042, Ibon Acherito, Pyrenees, 2004) obtained from the Institute of Zoology, Zoological Society of London. A single quantity sample of *Bsal* genomic DNA was used as a positive control. If any sample showed fluorescence growth in the wavelength of the *Bsal* probe, it would be re-analysed with the full set of *Bsal* standards. In this way, we slightly reduced the cost of analysis. In both detection assays, we used duplicates of all analysed samples, standards, as well as positive and negative controls.

All tested samples yielded negative results for the presence of *Bsal*. *Bd* was detected in three individuals of wild smooth newts and in one reared ribbed newt (*Pleurodeles waltl*) in a captive collection, albeit with no visible signs of the chytridiomycosis. Low *Bd* prevalence in caudates corresponds well with our previous findings in Czech captive collections (Havlíková et al. 2015), and wild populations of caudates in Central and east Europe (Baláž et al. 2014a,b, Vojar et al. 2017).

The 0% *Bsal* prevalence in wild caudates has Sterne-Wald 99% confidence limits of 0.0–4.2%, while in the case of samples from captivity the 99% confidence limits are 0.0–2.6% (Rósza et al. 2000). This does not directly mean that *Bsal* is not present in the Czech Republic. Because the disease outbreaks can occur at very low host densities in wild populations (Schmidt et al. 2017), all host populations of susceptible European caudate species (Martel et al. 2014) are at risk from *Bsal* (Schmidt et al. 2017). In the case of asymptomatic Asian caudates in captive collections, infection may be present in such small prevalence (Martel et al. 2014, Laking et al. 2017) that our sampling was not sufficient. On the other hand, because the intensive sampling of wild fire salamanders covered nearly all localities within Prague where the species presently is known to occur (Šťastný et al. 2015) and no sampled individual exhibited visible disease symptoms, we conclude that *Bsal* probably has not invaded Prague's fire salamander population, at least for now. Similar results of pathogen absence have been found in studies focused on fire salamanders in Austria (Gimeno et al. 2015), eastern hellbenders (*Cryptobranchus alleganiensis*) in the U.S. (Bales et al. 2015), Japanese giant salamanders (*Andrias japonicus*) in Japan (Bletz et al. 2017a), Chinese amphibians (Zhu et al. 2014), five species of newts and fire salamanders in most of tested localities in Belgium, Germany and the Netherlands (Spitzen-van der Sluijs et al. 2016), alpine newts, smooth newts and great crested newts in Germany (Bletz et al. 2017b), and in a study by Parrot et al. (2016) on 17 caudate species across three continents.

We used two available detection assays in our study, both based on DNA amplification with the same pair of *Bsal* primers (Blooi et al. 2013) and differing only in the detection format of the amplicon. The SYBR Green qPCR assay often produced detectable fluorescence growth of nonspecific products, thus complicating interpretation of the results. In several cases, we ran standard PCR followed by gel electrophoresis with samples of equivocal results to confirm the identity of PCR products. Our results indicated a mean melting temperature (Tm) for *Bsal* standards of 77.21°C (SD = 0.29), which differs slightly from the published value of 75.5°C (Blooi et al. 2013). For monitoring *Bsal* presence in wild and captive amphibians, we later adopted and recommend the use of duplex *Bd*+*Bsal* qPCR,

Table 2. List of surveyed species and numbers (No.) of individuals sampled in captivity.

| Species | No. | Species | No. |
|---|---|---|---|
| *Ambystoma mexicanum* | 3 | *Neurergus deryugina deryugina* | 2 |
| *Ambystoma tigrinum* | 2 | *Neurergus strauchii barani* | 3 |
| *Andrias davidianus* | 18 | *Neurergus strauchii strauchii* | 3 |
| *Calotriton asper* | 3 | *Ommatotriton ophryticus nesterovi* | 3 |
| *Cynops ensicauda ensicauda* | 3 | *Pachyhynobius shangchengensis* | 1 |
| *Cynops ensicauda popei* | 4 | *Pachytriton* sp. | 2 |
| *Cynops orientalis* | 1 | *Paramesotriton caudopunctatus* | 7 |
| *Cynops pyrrhogaster* | 3 | *Paramesotriton deloustali* | 6 |
| *Cynops pyrrhogaster* "Kanagawa" | 6 | *Paramesotriton guangxiensis* | 4 |
| *Cynops pyrrhogaster* "Yubana" | 2 | *Paramesotriton hongkongensis* | 3 |
| *Euproctus platycephalus* | 2 | *Paramesotriton chinensis* | 12 |
| *Hynobius dunni* | 1 | *Paramesotriton* sp. "helm" | 1 |
| *Hynobius leechii* | 2 | *Paramesotriton* sp. "red" | 6 |
| *Hynobius lichenatus* | 1 | *Paramesotriton yunwensis* | 2 |
| *Hynobius quelpartensis* | 2 | *Pleurodeles nebulosus* | 2 |
| *Hynobius retardatus* | 2 | *Pleurodeles waltl* | 4 |
| *Hypselotriton cyanurus* | 2 | *Salamandra algira tingitana* | 2 |
| *Hypselotriton cyanurus cyanurus* | 2 | *Siren intermedia* | 1 |
| *Hypselotriton chenggongensis* | 3 | *Triturus anatolicus* | 2 |
| *Hypselotriton orientalis* | 1 | *Triturus blasii* | 3 |
| *Ichthyosaura alpestris* | 3 | *Triturus carnifex* | 9 |
| *Laotriton laoensis* | 3 | *Triturus cristatus* | 2 |
| *Lissotriton boscai* | 3 | *Triturus dobrogicus dobrogicus* | 2 |
| *Lissotriton graecus* | 3 | *Triturus dobrogicus macrosoma* | 3 |
| *Lissotriton helveticus* | 3 | *Triturus ivanbureschi* | 1 |
| *Lissotriton italicus* | 3 | *Triturus karelinii* | 2 |
| *Lissotriton malcani* | 3 | *Triturus macedonicus* | 10 |
| *Lissotriton meridionalis* | 3 | *Triturus marmoratus* | 2 |
| *Lissotriton montandoni* | 3 | *Triturus pygmaeus* | 3 |
| *Neurergus crocatus complex* | 3 | *Tylototriton shanjing* | 2 |

which is designed specifically to detect either of the pathogen species (Blooi et al. 2013). The higher cost of analyses versus using fluorescent probes is counterbalanced by clearer and more specific results.

As not only newts, but also infected anurans and even waterfowl via scales on their feet, may promote fungal spread over large spatial distances (Stegen et al. 2017), the spread of this emerging pathogen is difficult to predict, and we can expect the distribution of *Bsal* to change considerably over time. The risk that new points of entry for *Bsal* into Europe will occur via the pet trade is a constant threat that can be alleviated by collaboration among pet owners, the pet trade, veterinary authorities, and conservationists (Sabino-Pinto et al. 2015). It is essential to prevent this pathogen entering the wild amphibian populations (Cunningham et al. 2015), because there is no effective method to reduce the impact of chytridiomycosis in the field (Garner et al. 2016). Therefore, our next planned steps in the Czech Republic include establishment and issue of biosecurity guidelines for owners of caudates, providing *Bsal* detection in captive collections of amphibians, forming a network of continuously monitored localities in proximity to larger cities, and preparing an action plan in case of *Bsal* occurrence in collaboration with the Nature Conservation Agency of the Czech Republic, the State Veterinary Authority, and the Czech Ministry of Environment.

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